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# Sp1-dependent activation of a synthetic promoter by human immunodeficiency virus type 1 Tat protein

**(transcriptional activation/Sp1 motif/GAL4–Tat fusion protein)**

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ABSTRACT **The Tat protein coded by human immunodeficiency virus (HIV) is a strong activator of viral gene expression from the long terminal repeat (LTR). It appears that Tat-mediated trans-activation of the HIV LTR is predominantly a transcriptional event. It has been reported that Tat acts at the level of both transcriptional initiation and elongation through interaction with a nascent RNA target sequence termed TAR (for trans-activation response element). However, the precise mechanism(s) by which Tat mediates TAR-dependent transcriptional activity is not known. To determine whether Tat functions similarly to other eukaryotic transcriptional activators through any of the conventional promoter elements, we tested Tat activity on synthetic promoters containing consensus sequences required for binding transcription factor Sp1 and a TATA box. Here, we report that a chimeric Tat protein targeted to the promoter region by the DNA-binding domain of yeast transcription factor GAL4 activates the synthetic promoter. Because this trans-activation depends on Sp1-binding sites, Tat can apparently mediate transcriptional activation through its interaction with Sp1. Mutational analysis of the *gal4–tat* chimeric gene reveals that the N-terminal 48-amino acid region of Tat constitutes the activation region for Sp1-dependent trans-activation. This region of Tat exhibits substantially more activity than the N-terminal 58 amino acids of Tat, which includes the arginine-rich basic region. Effects of specific mutations in the 48-amino acid Tat region of GAL4–Tat on trans-activation of the synthetic promoter mimic the effects of these specific mutations on Tat-mediated trans-activation of the HIV-1 LTR, suggesting that trans-activation of both the synthetic promoter and the intact LTR occurs by a common mechanism.**

The *tat* gene of human immunodeficiency virus (HIV) plays a central role in the life cycle of HIV because it is essential for viral replication (1, 2). The *tat* gene of HIV-1 codes for a nuclear protein (Tat) of 86–103 amino acids, depending on the HIV-1 strain (3). The Tat protein is a strong activator of viral gene expression from the long terminal repeat (LTR) (4, 5) and functions through a trans-activation response element (TAR) located downstream of the transcription initiation site (6). A number of studies suggest that trans-activation by Tat is predominantly a transcriptional event (for review, see refs. 7 and 8). Tat has been reported to act at the level of both transcriptional initiation and elongation (9–13); however, the mechanism by which Tat mediates this TAR-dependent transcriptional activity is unknown. For example, an important unanswered question is whether Tat functions directly or indirectly through a specific DNA promoter sequence as for various other eukaryotic transcriptional activators.

It is now accepted that TAR functions as an RNA target rather than as a DNA target (14–16). Because purified Tat protein can specifically bind to TAR RNA *in vitro* (17–21, 52), it appears that Tat-mediated trans-activation involves direct RNA (TAR)–protein (Tat) interaction. Recent studies suggest that the sole function of TAR might be to target Tat to the HIV promoter. In these studies the HIV-1 TAR sequences were replaced with the target sequences of heterologous RNA-binding proteins and the HIV promoter trans-activated by Tat fusion proteins containing the cognate RNA-binding domains (22, 23). However, how the Tat protein placed in the vicinity of the LTR activates transcription is unclear.

Although HIV-1 LTR contains a number of regulatory elements and has been shown to interact with several cellular factors (53), previous mutational analyses have delineated two major promoter elements within the LTR: a core promoter region required for LTR expression in most cell types and an enhancer region that appears important for LTR expression in activated T cells (24). The core promoter elements contain three binding sites for the transcription factor Sp1 and a TATA region (6, 9, 25–27). Mutational analyses have indicated that the core promoter region is essential for both the basal and Tat-induced activity of the LTR (6, 9, 26, 28). It has been difficult to define the role of core promoter elements (Sp1 and TATA) in Tat-induced activation because mutations to these motifs have deleterious effects on basal promoter activity. To determine whether Tat functions in a manner similar to eukaryotic DNA sequence-dependent transcriptional activators through its interaction with core promoter elements, we have used synthetic promoters consisting of either the TATA region alone or the TATA region and Sp1-binding sites. The use of minimal synthetic promoters might allow the positive identification of specific elements that are sufficient for Tat-mediated trans-activation. The minimal promoter (e.g., adenovirus E1b TATA) approach has been elegantly used to study the mechanisms of trans-activation by various transcriptional activators, including acidic-blob activators such as GAL4, VP16 (29), and adenovirus E1a (30). In these studies, activators were targeted to the promoter by using a fusion-protein strategy in which the activator was linked to the DNA-binding domain of the yeast transcription factor GAL4 (31). We have used a GAL4–Tat fusion-protein approach to determine whether Tat could function like a typical DNA sequence-dependent transcriptional activator and also to identify the target for Tat-mediated trans-activation. Here, we report that trans-activation by GAL4–Tat depends on Sp1-binding motifs and identify the activation region of Tat required for the Sp1-dependent activation.

## MATERIALS AND METHODS

**Plasmids and Mutants.** Reporter plasmids pLTR–chloramphenicol acetyltransferase (CAT) (26), pBCAT, and

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Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; TAR, trans-activation response element.

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pG5BCAT (30) have previously been described. Plasmids pBCAT–SP and pG5BCAT–SP were constructed by cloning a double-stranded oligonucleotide containing three consensus binding sites (5′-GGGGCGGGGC-3′) for transcription factor Sp1 (32) at the *Xba* I site immediately 5′ to the E1b TATA box. The plasmid expressing the GAL4 DNA-binding domain (pSG424, ref. 33) and pTAT expressing the 101-amino acid version of HIV-1 (strain SF2) Tat protein (26) have been described. Both plasmids use the simian virus 40 early promoter for expression. Plasmid pSG424 is referred to as pGAL4 in the text and figures. The *gal4–tat* chimeric gene was constructed by cloning a *Bgl* II (blunt-ended with mung bean nuclease)–*Sac* I DNA fragment from pTAT between *Sma* I and *Sac* I sites of pSG424.

Mutant *gal4–tatΔ1-7* was constructed by cloning an *Xba* I (blunt-ended)–*Sac* I fragment from pTAT between the *Bam*HI (blunt-ended) and *Sac* I sites of pSG424. Mutants *gal4–tat(22)*, *gal4–tat(30)*, *gal4–tat(37)*, *gal4–tat(41)*, and *gal4–tat48* were constructed by substituting an *Avr* II–*Sac* I DNA fragment from mutants *tat22*, *tat30*, *tat37*, *tat41*, and *tat48* that have been described (34). Tat 101(22) has a Cys-22→Gly mutation and serves as a negative control.

**Trans-Activation.** HeLa cells (100-mm$^2$ dishes) were transfected with various reporter plasmids (2 μg) and activator plasmids (2 μg) along with salmon sperm carrier DNA (20 μg) by the calcium phosphate method. Forty-eight hours after transfection, cell extracts were assayed for CAT activity (35). Unacetylated and acetylated forms of [$^{14}$C]chloramphenicol were separated by thin-layer chromatography and were quantitated by scraping the radioactive spots and scintillation counting.

## RESULTS

**Effect of GAL4–Tat on Synthetic Promoters.** To determine whether either of the two core promoter motifs are targets for Tat, we first tested the ability of GAL4–Tat to trans-activate a minimal promoter comprising of a prototypical TATA region (E1b TATA) (30) and five GAL4-binding sites (36) linked to the bacterial CAT reporter gene (pG5BCAT, Fig. 1*a*). The E1b TATA sequence has previously been shown to be activated by a number of TATA box-dependent eukaryotic transcriptional activators such as GAL4, VP16, E1a, and p53. In these studies a *gal4–tat* chimeric gene (pGAL4–Tat) expressing the DNA-binding domain (N-terminal 147 amino acids) of GAL4 (37) and the N-terminal 58 amino acids of HIV-1 Tat was used as the activator (Fig. 1*b*). Previous mutational studies of the HIV-1 *tat* gene have indicated that the N-terminal 58 amino acids of Tat constitute the essential region for Tat activity (see ref. 34 and the references therein). HeLa cells were cotransfected with the reporter plasmid pG5BCAT and with pGAL4–Tat, and CAT activity was determined. As seen in Fig. 1*c*, pGAL4–Tat did not appear to have any detectable effect on the expression of pG5BCAT. Activity of the *gal4–tat* chimeric gene was then tested on a reporter containing the E1b TATA region and the Sp1-binding sites (pG5BCAT–SP, Fig. 1*a*). Cells cotransfected with pGAL4–Tat and the reporter plasmid pG5BCAT–SP exhibited ≈20-fold trans-activation compared with cells cotransfected with pG5BCAT–SP and the control plasmid pGAL4 (Fig. 1*c*). Further, the activation depended on the GAL4 targeting system because pGAL4–Tat was inactive on pBCAT–SP, which lacks the GAL4-binding sites (Fig. 1*c*). Similarly the native Tat protein (Tat 101) was inactive on pG5BCAT–SP, which lacks the cognate TAR element (see Fig. 4). Because the CAT activity expressed by pG5BCAT was near the limits of detection in these CAT assays, dose–response transfections were done with pG5BCAT DNA (Fig. 2). Four, eight, and sixteen micrograms of pG5BCAT DNA were cotransfected with pGAL4 or pGAL4–Tat48. GAL4–

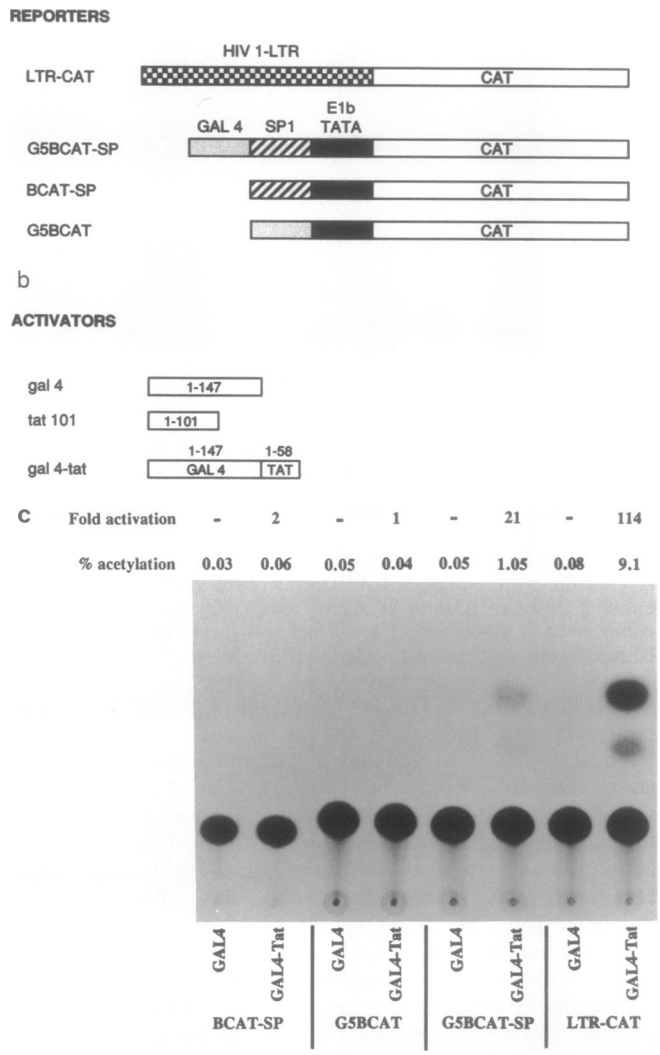


FIG. 1. Schematic diagrams of reporter (*a*) and activator (*b*) constructs and activation of reporter constructs by GAL4–Tat fusion protein (*c*). CAT assays were done as described. For determining fold trans-activation, percent acetylation induced by pGAL4 is taken as 1.

Tat48 is a GAL4–Tat derivative that lacks the basic domain of Tat and shows increased activation of CAT expression from pG5BCAT–SP (see Fig. 4). Although the level of CAT activity from pG5BCAT is very low, CAT activity definitely increases as the amount of pG5BCAT DNA is increased from 4 to 8 and 16 μg of DNA (corresponding to 0.02, 0.039, and 0.063% acetylation, respectively) in the transfection assays. The basal CAT activity with 16 μg of pG5BCAT DNA is equivalent to the CAT activity of 2 μg of pG5BCAT–SP (0.067% acetylation). In these assays, cotransfection of pGAL4–Tat48 resulted in ≈80-fold activation of CAT activity of pG5BCAT–SP. However, no activation of CAT expression by pGAL4–Tat48 was seen at any concentration of pG5BCAT (Fig. 2). We conclude that the basal level of CAT expression from pG5BCAT, although very low, is measurable and that pGAL4–Tat48 is completely inactive on the synthetic promoter containing only the TATA box. This result indicates that Tat may not have a transcriptional activation function similar to various activators that mediate their effect through the TATA region alone. Similarly, a TATA region derived from the HIV-1 LTR was also not trans-activated by pGAL4–Tat, whereas it could be effi-

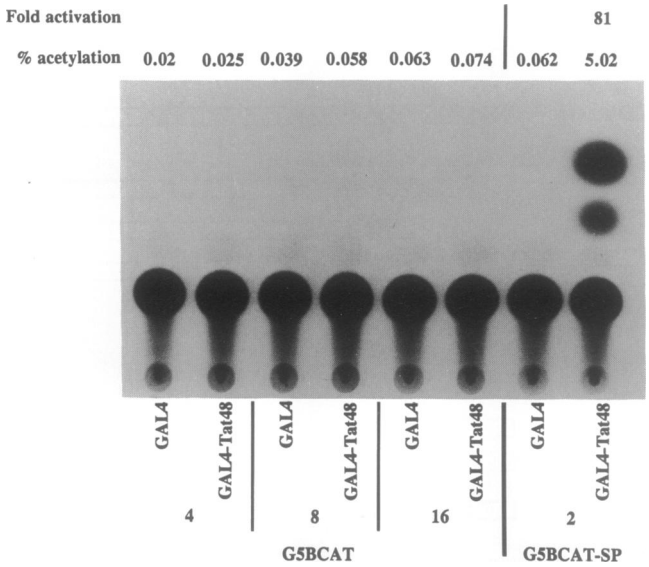


FIG. 2. Dose–response of pG5BCAT DNA. Four, eight, and sixteen micrograms of pG5BCAT DNA were each cotransfected with either 2 μg of pGAL4 or pGAL4–Tat48 DNA, as described. For comparison, 2 μg of pGAL4 and pGAL4–Tat48 DNA were each cotransfected with 2 μg of pG5BCAT–SP DNA.

ciently activated by GAL4–VP16 (38) (T.S. and G.C., unpublished results).

These results indicate that Tat directed to the promoter containing Sp1-binding motifs via the GAL4 DNA-binding sites has a sequence-specific trans-activation function and this activity depends on Sp1 motifs. The level of trans-activation of pG5BCAT–SP by pGAL4–Tat is, in general, about one-tenth the level seen with pLTR–CAT and pGAL4–Tat (Fig. 1*c*). This result would be expected because the synthetic promoter lacks the Tat–TAR targeting system that may be more efficient than the GAL4–Tat targeting system and/or due to absence of other promoter elements that may augment the overall promoter activity of the LTR.

**Effect of Tat Mutations on Trans-Activation of Sp1–TATA Promoter.** To determine whether the functional domains of Tat required for Sp1-dependent activation and for the activity of the native *tat* gene are the same, we transferred single amino acid substitutions or deletion mutations from the native *tat* gene to the *gal4–tat* gene. For better comparison, we studied the effects of specific mutations which have previously been evaluated in the native *tat* gene background (ref. 34; Fig. 3). Previous mutational studies have indicated that the N-terminal 58-amino acid region of Tat is essential for trans-activation of the LTR (34, 39–42). This 58-amino acid region has been tentatively divided into four domains (34), as illustrated in Fig. 3*a*. The N-terminal region contains three acidic residues and is predicted to be capable of forming an amphipathic helix similar to eukaryotic acid-blob activators (41). Previous mutational studies of the *tat* gene have indicated that mutations within the N-terminal region have various effects on Tat activity (34, 39–43). Mutant *gal4–tatΔ1-7* contains a deletion of the N-terminal seven residues of Tat, which deletes two of these three acidic residues (domain A in Fig. 3*a*). Mutants *gal4–tat(22)*, *gal4–tat(30)*, and *gal4–tat(37)* (Fig. 3*b*) have single-amino acid substitutions (Cys→Gly) in three of the seven cysteine residues (domain B), which are highly conserved among the Tat proteins coded by various primate and HIVs. These cysteine residues that are essential for Tat activity (34, 39–42) have been implicated in metal-linked dimerization of the Tat protein (44). Mutant *gal4–tat(41)* contains a threonine substitution for a conserved lysine residue (position 41) in domain C. We have previously

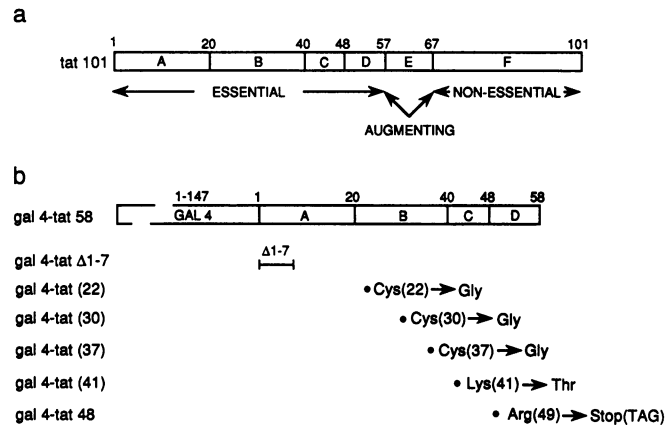


FIG. 3. (*a*) The tentative domain map of HIV-1 Tat is based on previous mutational analysis of the native *tat* gene of HIV-1, strain SF2 (34). (*b*) Organization of various *gal4–tat* mutants.

shown that this lysine residue is crucial for Tat function (34). Mutant *gal4–tat48* contains a chain-termination mutation at codon 49 and can only express the N-terminal 48 amino acids of Tat and, hence, lacks the basic domain (domain D). The arginine-rich basic domain has been shown to mediate sequence-specific binding with TAR RNA (17–21, 52). The activity of these mutants was tested on the reporter plasmid pG5BCAT–SP (Fig. 4). All mutants in domains A–C appear functionally inactive, whereas mutant *gal4–tat48*, which lacks the basic domain induced CAT expression (60-fold) to levels that even exceed that of the wild-type *gal4–tat58* (29-fold, Fig. 4). As expected *gal4–tat48* was almost completely inactive on the HIV-1 LTR–CAT, which lacks the GAL4-binding sites but contains TAR (Fig. 4). These results indicate that domains A–C are essential, whereas the basic domain is not essential for Sp1-dependent transcriptional activation by Tat. Because the primary function of the arginine-rich basic domain appears to be interaction with TAR RNA (17–21), the basic domain would not be expected to be essential in the GAL4-targeting system.

The inactivity of the various GAL4–Tat mutants is not concentration dependent. Fig. 5*a* is a dose–response experiment comparing pGAL4–Tat48 and gal4–tat(41). Activation of CAT expression from pG5BCAT–SP could be detected at 0.2 μg of pGAL4–Tat48 DNA; however, no activation of CAT expression could be detected at a 30-fold higher concentration of *gal4–tat*(41) DNA. Although gal4–tat(41) activity could not be detected in these assays, the presence of gal4–tat(41) protein could be inferred from blocking of pGAL4–Tat48 activation by gal4–tat(41). Cotransfection of a 6-fold excess of *gal–tat*(41) DNA to pGAL4–Tat48 DNA resulted in an almost complete elimination of pG5BCAT–SP activation by pGAL4–Tat48 (Fig. 5*b*). In other experiments (data not shown), we have found that cotransfection of pGAL4 can inhibit activation by pGAL4–Tat48, indicating that all inactive GAL4–Tat derivatives should be trans-dominant in the GAL4 targeting system. This suggests that the reason defective *tat* mutants are not trans-dominant on the LTR (42) is because TAR RNA is not an easily saturable target; this most likely is due to the presence of multiple copies of TAR RNA at the promoter or the ability of the TAR RNA to turn over. Antiviral strategies relying on a trans-dominant Tat phenotype or on inactivation of the TAR RNA target might not be successful for this reason.

Our mutational analyses of GAL4–Tat agree very well with the previous mutagenesis studies of the native *tat* gene and suggest that the domains required for trans-activation by native Tat and GAL4–Tat are identical. Thus, the region of native Tat located within the N-terminal 48 amino acids is

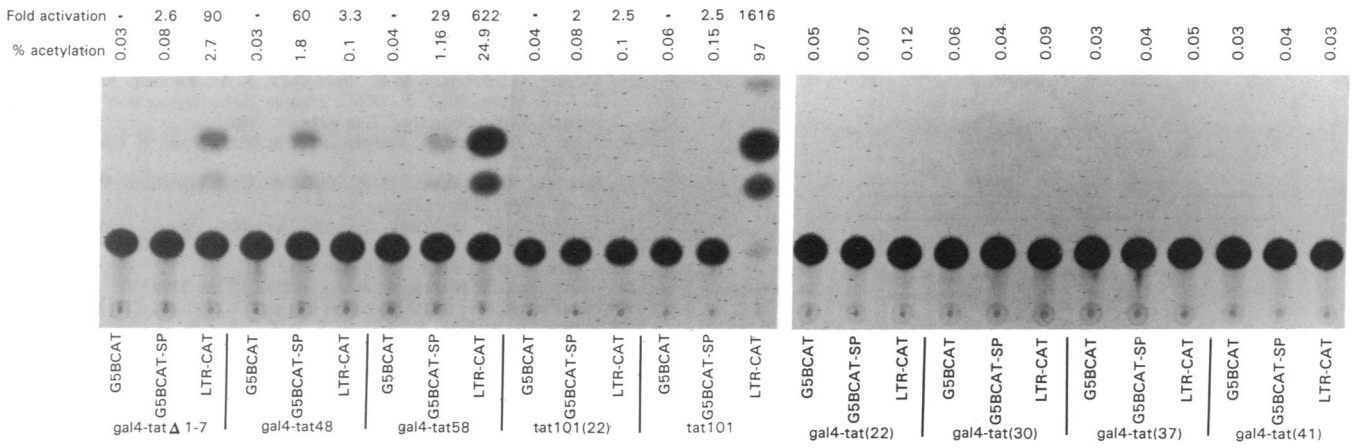


FIG. 4. Effect of *gal4–tat* mutations. The effect of various mutants of the *gal4–tat* gene on trans-activation of pB5CAT–SP was determined as in Fig. 1. Fold activation by different Gal4–Tat constructs on pG5BCAT–SP and pLTR–CAT is expressed as percent acetylation on these reporter constructs compared with pG5BCAT.

also required for the activity of GAL4–Tat. This region appears to constitute the activation region of Tat required for Sp1-dependent trans-activation. We note that *gal4–tatΔ1-7*, which was inactive on pG5BCAT–SP, induced low-level CAT activity (≈15% of wild-type *gal4–tat58*) on pLTR–CAT, which contains the entire HIV-1 LTR. This is not surprising considering the various effects observed with N-terminal Tat mutants in the context of entire HIV-1 LTR (42, 43).

## DISCUSSION

Our results indicate that Tat can function in a manner similar to a typical eukaryotic transcriptional activator through a specific promoter element. Because this activation depends on Sp1-binding sites, Tat may function in concert with transcription factor Sp1. Although the N-terminal acidic region of Tat appears essential for the trans-activation, our results seem to rule out the suggested possibility (41) that Tat may function like various acid-blob activators because pGAL4–Tat does not trans-activate promoters containing only a TATA box (Figs. 1*c* and 2). It has also been suggested that Tat can function as a DNA sequence-dependent trans-activator from studies done with a Tat–Jun fusion protein and an LTR construct containing AP1 DNA-binding sites located downstream of the transcriptional start site within TAR (28). Although the Tat–Jun fusion protein could be selectively targeted to the HIV promoter, the AP-1 site had to be positioned downstream of the transcriptional start site. Because transcriptional activators that interact with the core promoter elements can be targeted upstream of the start site, whether Tat functions through a core promoter element was uncertain.

From mutational studies, promoter elements located within an area spanning the NF-κB and Sp1 motifs were identified as the targets for Tat-induced activation (28). Besides Sp1 (25), this region has been reported to interact with NF-κB (45–47) and a factor termed EBP-1 that appears distinct from NF-κB (48). It was unclear whether any of these three factors or other novel factor(s) were involved in Tat-induced trans-activation. Although our studies do not exclude other target sites for Tat-induced activation, the use of a synthetic promoter consisting of consensus Sp1-binding sites and TATA box has enabled us to specifically identify the Sp1 motif as a target for Tat-induced trans-activation. Furthermore, we have shown that the GAL4–Tat targeting system is sensitive to a variety of Tat mutations that normally inactivate Tat activity on the HIV-1 LTR, implying that Sp1-dependent activation seen with the minimal promoter is an authentic functional component of Tat trans-activation. Our results also indicate that a downstream targeting signal at TAR is not essential for Tat activity and the Sp1-TATA synthetic promoter can be activated from an upstream targeting site as for other eukaryotic transcriptional activators.

We do not know whether Tat interacts with Sp1 directly or indirectly. It has recently been postulated that Sp1 activity is mediated by a coactivator that functions as an adapter to connect Sp1 to the basic transcriptional machinery consisting of TATA–TFIID promoter complex (49). However, no Sp1

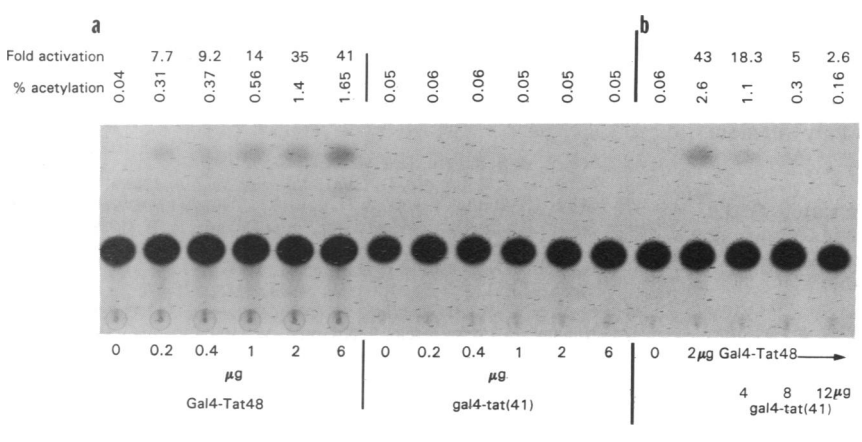


FIG. 5. (*a*) Dose–response of pGAL4–Tat48 and gal–tat(41) DNA. Increased amounts (μg) of pGAL4–Tat48 and gal–tat(41) DNA were co-transfected with 2 μg of pG5BCAT–SP DNA. (*b*) Blocking of pGAL4–Tat48 activation by gal–tat(41). pG5BCAT–SP DNA was cotransfected with 2 μg of pGAL4 (lane 0) or pGAL4–Tat48 DNA. Effect of increased amounts (4, 8, and 12 μg) of gal4–tat(41) DNA on GAL4–Tat48 activation of pG5BCAT–SP was determined. To control for promoter competition, pGAL4 DNA was cut with *Bgl* II and included in the transfection to bring combined total of gal–tat(41) competitor DNA and pGAL4 *Bgl* II-digested DNA to 12 μg. *Bgl* II cuts pGAL4 DNA immediately after simian virus 40 promoter, so no GAL4 sequences can be expressed. Fold activation of pG5BCAT–SP by GAL4–Tat48 is percentage acetylation compared with percentage acetylation induced by pGAL4.

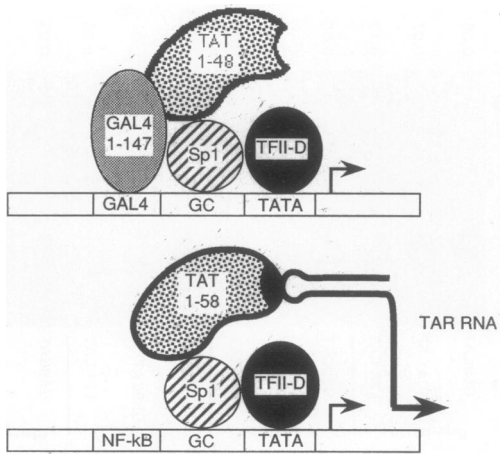


FIG. 6. Models for transcriptional activation by HIV-1 Tat proteins. (*Top*) Activation of minimal promoter containing Sp1 and TATA motifs by GAL4–Tat. The activation region (N-terminal 48 amino acids) of Tat is targeted to the Sp1–TATA minimal promoter through the GAL4 targeting system. (*Bottom*) Activation of HIV-1 LTR by native Tat. Tat is targeted to the LTR through interaction of TAR with the basic domain of Tat (filled area within Tat). GC, consensus binding sites.

cofactor has yet been identified. Because our functional assay indicates that Tat activity depends on Sp1, Tat could function as a coactivator of Sp1. Our results show that Tat does not possess an activity similar to the acidic-blob activators, as the minimal TATA promoter is not activated by GAL4–Tat, whereas it can be efficiently activated by a GAL4–VP16 chimeric gene product. Clearly, more studies are required to elucidate the precise mode of interaction of Tat with Sp1 and the basic transcriptional machinery. Because the minimal promoter used here contains only the Sp1 and TATA motifs, Tat may primarily modulate the core Sp1–TATA promoter activity by direct or indirect interaction with Sp1. A tentative model based on our results with the synthetic promoter is illustrated in Fig. 6. In this model GAL4 (1–147) serves a simple targeting function for Tat via GAL4-binding sites in place of the TAR sequence downstream of the HIV promoter and the Tat basic domain. In either instance targeted Tat is postulated to interact with bound Sp1 to activate transcription.

Because trans-activation of the minimal promoter construct described here requires Sp1, which is a component of the transcriptional initiation machinery (50), at least one of the activities of Tat may be at the level of transcriptional initiation. Several recent reports have suggested that Tat may increase the rate of transcriptional elongation as well as the rate of initiation (11–13). Perhaps effects on transcriptional initiation and elongation may be coupled so that interaction of Tat with initiation factors such as Sp1 induces alterations in the basal transcription complex important for efficient elongation. Recently, examples of conditional blockage of transcription elongation have been reported for eukaryotic genes, along with evidence that modifications to eukaryotic RNA polymerase II transcription complexes may regulate transcriptional elongation (51).

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